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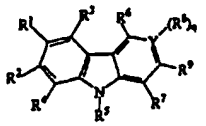
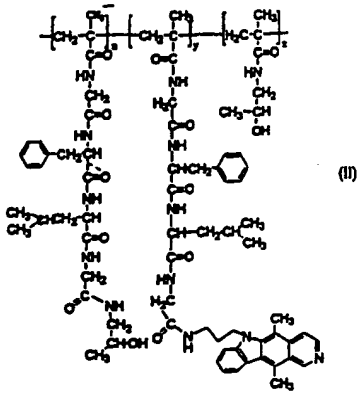
(54) Title: CONJUGATES OF HPMA COPOLYMER AND ELLIPTICIN



(I)



(II)

(57) Abstract: Polymer and copolymer conjugates of a bioactive heterocyclic compound, the general structure of which is represented by formula (I). In a preferred embodiment 6-(3-aminopropyl)-ellipticine (APE) is linked to a copolymer via pendent, lysosomally labile peptide side-chains. The bioactive agent is linked to the polymer using aminolysis of carboxyterminal p-nitrophenyl ester groups represented by formula (II) (Ulbrich et al., 1996).

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

CONJUGATES OF HPMA COPOLYMER AND ELLIPTICIN

The present invention is concerned with drug targeting using polymer-bioactive conjugates, novel conjugates and the production thereof.

Background of the Invention

In recent years there has been a great deal of investigation of polymers as carriers of anticancer drugs. Polymer-drug conjugation is known to have the potential to improve tumour targeting and reduce the concentration of drug in sites of systemic toxicity (Duncan, 1992; Duncan et al., 1996)[1,2]. For example, HPMA copolymer-doxorubicin delivered significantly greater levels of doxorubicin (measured as AUC) to s.c. B16F10 tumours than could be achieved by bolus injection of free doxorubicin (Seymour et al., 1994)[3]. The cardiac toxicity of doxorubicin, at doxorubicin equivalent doses, is much diminished by attachment of the drug to the HPMA copolymer (Young et al., 1991)[4] while intratumoural release of the drug is ensured by lysosomal enzymatic degradation of the Gly-Phe-Leu-Gly peptide side chain (in the PK1 conjugate) by thiol-dependent proteinases (Duncan, 1992)[1].

The basis for much of this work is that attachment of toxic drugs to high molecular weight carriers can lead to reduction in systemic toxicity, longer retention time in the body, alterations in biological distribution, improvements in therapeutic efficacy and site specific passive capture through the enhanced permeability and retention (EPR) effect. The EPR effect results from enhanced permeability of macromolecules or small particles within the tumour neovasculature due to leakiness of its discontinuous endothelium. In addition to the tumour angiogenesis (hypervasculature) and irregular and incompleteness of vascular networks, the attendant lack of lymphatic drainage promotes accumulation of macromolecules that extravasate. This effect is observed in many solid tumours for macromolecular agents and lipids. The enhanced vascular permeability will support the great demand of nutrients and oxygen for the rapid growth of the tumour. Unless specifically addressed for tumour cell uptake by receptor-mediated endocytosis, polymers entering the intratumoural environment are taken up relatively slowly by fluid-phase

pinocytosis. Many polymer-based anticancer agents have now entered the clinic or are passing through clinical trials; each has proven the concept compared to the native drug. For instance, N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-doxorubicin conjugates have already shown promise in early clinical trial. Moreover, residual HPMA copolymer conjugate which does not permeate into the tumour but remains in the circulation is rapidly excreted giving a high tumour:blood ratio.

These conjugates are a subset of a class of compounds known as "Polymer Therapeutics". Polymer therapeutics are taken up into a cell by pinocytosis where the drug is liberated in the lysosome. The lysosomal pH is 5.5 compared to 7.4 in circulating blood, and lysosomes contain a vast array of hydrolytic enzymes including proteases, esterases, glycosidases, phosphates and nucleases. Hence drugs have been conjugated to polymers using conjugation linkers that degrade in the lysosome while remaining intact in the bloodstream. Since many drugs are not pharmacologically active while conjugated to a polymer, this results in drastically reduced toxicity compared to the free drug in circulation. The relatively harsh environment of the lysosome has thus inspired the development of conjugation linkers that degrade only in the lysosome to release the drug ("lysosomotropic drug delivery").

A wide variety of linkages have been used to covalently bind a drug to the polymeric carrier. Several examples include amide, ester, hydrazide, urethane, carbonate, imine, thioether, azo and carbon-carbon bonds.

Following the concept of lysosomotropic drug delivery, peptidyl linkers designed to be stable in the bloodstream, but degradable by lysosomal enzymes and thus able to release the drug intracellularly, have been investigated.

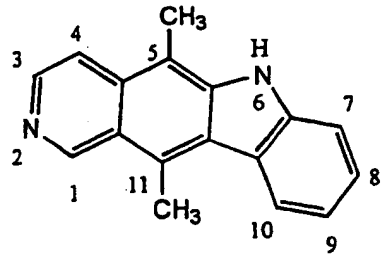
Peptide linkers have been shown to mediate lysosomotropic drug delivery. It has become apparent that one of the methods of control of the rate and location of drug release from pendent chain polymers is favourably effected when a drug is bound to the polymer backbone via a peptidyl side-chain.

Since the discovery that peptidyl side chains in HPMA (hydroxypropylmethacrylamide) copolymers could be designed for cleavage

by model enzymes such as chymotrypsin, trypsin and papain, recent studies have seen the systematic development of HPMA copolymer-anticancer conjugates containing peptidyl linkers tailored for cleavage by lysosomal proteases. Such linkers have now become more widely used in many different polymer conjugates.

Several tetracyclic compounds have shown antitumour activity. Particularly promising compounds include ellipticine and derivatives thereof, 9-azaellipticines and derivatives thereof, pazellipticine, ditercalinium and intoplicine.

The alkaloid ellipticine below, 5,11-dimethyl-6H-pyrido(4,3-b)carbazole, is a potent cytotoxic agent whose mechanism of action has been attributed variously to direct intercalation into DNA, to modification of topoisomerase activity or to alkylation of cellular DNA or other macromolecules. Alkylation may be mediated through formation of the 9-hydroxy-metabolite generating thereafter an iminoquinone electrophile or oxidation and subsequent phosphorylation of the 5-methyl group whose presence is known to contribute to drug activity. The 9-OH derivative of ellipticine has a cytotoxicity activity 40 times that of ellipticine itself. Its preparation is described in EP-A-0009455.



The early promise of ellipticine and its derivatives as useful anti-cancer drugs has been limited by their relative insolubility in physiological fluids and compromised by host toxicities encountered *in vivo*, notably rapid hemolysis and decreased heart rate in mammals (Herman et al., 1971; Herman et al., 1974a; Herman et al., 1974b; Donato et al., 1992[5,6,7,8]). A more soluble derivative, the quaternary salt 9-hydroxy-2-methylellipticinium acetate (below), has been disclosed to be efficacious against human thyroid

and renal tumours (Rouesse et al., 1981[9]) and against bone metastases associated with breast cancer (Rouesse et al., 1993[10]) other ellipticinium derivatives wherein a quaternising alkyl group has an amine substituent have been described in EP-A-0209511, FR-A-2527209 and US 4,310,667.

In WP-A-9200315 9-amino ellipticine is conjugated to an oligo nucleotide by reaction with a phosphoramidite reagent.

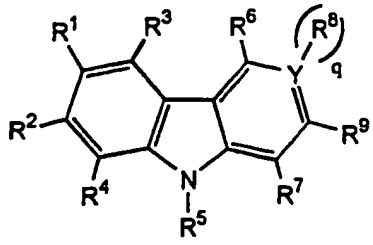
In EP-A-0608876 ester derivatives of the 9-hydroxy ellipticine derivatives are described. The acyl moiety may be of an amino acid and hence include primary amine substituents.

In EP-A-0591085 ellipticine derivatives have alkylaminocarboxyl substituents at the 1-carbon atom. The alkyl group has an amine group substituent.

Analysis of ellipticine having conjugated unsaturated heterocyclic (N-containing) ring structures with a generally planar conformation have been described with similar activity. Such compounds include ditercalinium and intoplicine. Intoplicine has an aminoalkyl amine substituent whilst ditercalinium is a pyridinium compound in which the quaternising alkyl group is substituted by a N-nitrogen heterocycle.

Summary of Invention

This invention comprises novel polymer and copolymer conjugates of a bioactive heterocyclic compound, the general structure of which is represented by the structure (I)



(I)

wherein Y is either nitrogen or carbon, each of R^1 to R^9 are selected from the group consisting of QP, hydrogen, hydroxyl, -$CONH_2$, cyano amino, halogen, glycosyl, (di)alkyl amino, C_{1-4} alkoxy, C_{1-12} alkyl, C_1 - C_{12} alkenyl, C_6 - C_{30}, aryl, C_7 - C_{30} aralkyl, C_{7-30} alkaryl, C_3 - C_{30} cycloalkyl, C_{2-6} alkanoyloxy and C_{7-10} aralkanoyloxy groups any of which alkyl or aryl groups

may be unsubstituted or substituted with a group selected from the the group consisting of carboxy, amine (including (di)alkyl amino) acyl, acyloxy, acylamino, alkoxy, hydroxy; R^1 and R^2 or R^1 and R^3 may together with the carbon atoms to which they are joined form an aromatic 6-membered substituted or unsubstituted carbocyclic or heterocyclic ring or, if Y is carbon, R^6 and R^8, or R^8 and R^9 may, together with the carbon atoms to which they are joined, form an aromatic carbocyclic or heterocyclic 6-membered ring, provided there is at least one 6 membered nitrogen-containing heteroaromatic ring in the molecule, and provided that one and only one of R^1 to R^9 is -QP, Q is a linker group and P is a polymer having a molecular weight in the range 100D to 800KD which is water-soluble; when Y=carbon, q=1 and when Y=nitrogen q=0 or 1. The components of the polymeric backbone may comprise acrylic polymers, alkene polymers, urethane polymers, amide polymers, polyimines, polysaccharides and ester polymers. Preferably the polymer is synthetic rather than being a natural polymer or derivative thereof. Preferably the backbone components comprise derivatised polyethyleneglycol and poly(hydroxyalkyl(alk)acrylamide), most preferably amine derivatised polyethyleneglycol or hydroxypropyl(meth)acrylamide-methacrylic acid copolymer or derivative thereof. A preferred molecular weight range is 15 to 40 kD.

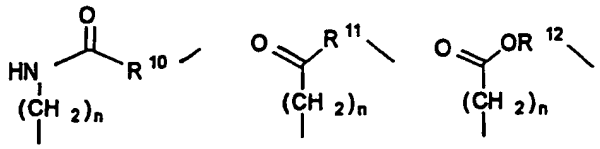
Preferably, where Y is nitrogen, q is 0.

R^4, R^6, R^8, and R^7 are preferably selected from the group consisting of hydrogen, hydroxyl, and C_1 - C_{12} alkyl, more preferably hydrogen, methyl, ethyl, propyl and butyl, most preferably hydrogen or methyl.

Where Y is carbon it is preferred for R^6 and R^7 each to be methyl, R^1 is preferably hydrogen, hydroxy or acyloxy (preferably C_{2-6}-alkanoyloxy), R^2 and R^3 are preferably hydrogen.

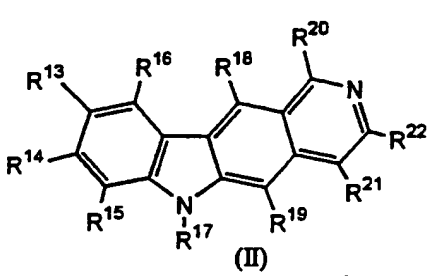
Preferably, R^5 comprises a group selected from the group consisting of hydrogen, C_1-C_6 alkyl but is most preferably QP. The bioactive heterocycle utilised in the present invention may be attached to the polymeric backbone via any of the groups R^1-R^9 i.e. any of R^1 to R^9 may represent QP. Preferably the bioactive heterocycle is attached to the polymeric backbone via QP.

QP, for example, may comprise a cleavable peptide linkage most preferably a peptide linkage capable of being cleaved by preselected cellular enzymes, for instance, those found in lysosomes of cancerous cells. Alternatively, an acid hydrolysable linker could comprise an ester or amide linkage and be for instance, a cis-aconityl linkage. The linker group may comprise a structure selected from the group consisting of

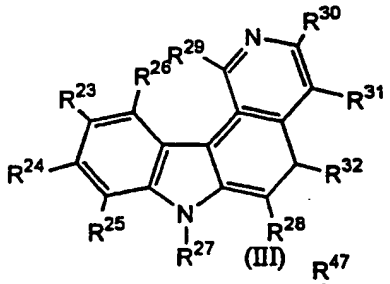


wherein n is an integer of 0-30, R^{10}-R^{12} are selected from the group consisting of, C_1-C_{12} alkanediyl, C_{2-12} alkenylene and C_6-C_{18} arylene.

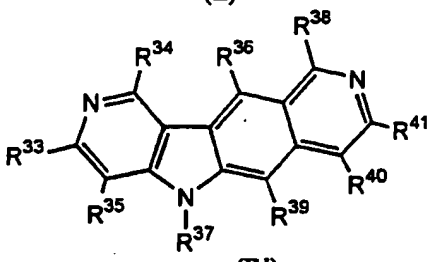
Some preferred bioactive heterocycles of the present invention include the following structures, (II)-(V):



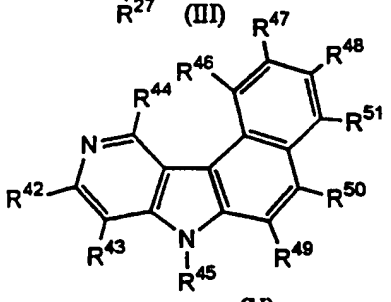
(II)



(III)



(IV)



(V)

Wherein R^{13}-R^{51} are selected from the respective groups listed above as being represented by R^1. A particularly preferred heterocycle has structure (II).

In the compound of the formula (II), R^{18} and R^{19} are preferably both methyl. R^{13} is preferably hydrogen, hydroxyl, alkoxy or alkanoyloxy. R^{14},

R^{15}, R^{16}, R^{20}, R^{21}, and R^{22} are preferably each hydrogen, and R^{17} is preferably QP as defined hereinbefore.

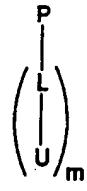
In compounds of the general formula (III), R^{23} is preferably hydroxy or alkoxy, preferably methoxy. R^{24} to R^{26} and R^{28} to R^{32} are preferably all hydrogen, R^{27} is preferably QP.

In compounds of the general formula (IV) R^{36} is preferably hydrogen. R^{33} to R^{35}, R^{37} and R^{39} to R^{41} are preferably hydrogen. R^{38} is preferably hydrogen or substituted C_{2-4} alkyl amino, for instance (N, N-dialkylamino)alkyl amino, such as 3-(N, N-dimethylamino)propylamino, and R^{37} is preferably QP.

In compounds of the formula (V), R^{48} is preferably hydroxyl or C_{1-4} alkoxy. R^{44} is preferably hydrogen or substituted C_{2-4} alkyl amino, such as (3-N, N-dimethylamino)propyl amino. R^{45} is preferably QP.

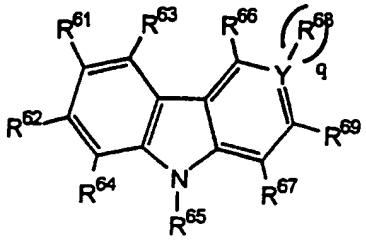
Preferably the bioactive heterocycle utilised in the present invention is attached to the polymeric backbone via the 5-membered ring nitrogen of any of structures (II)-(V), or by R^{44} of structure (V) and R^{13} or R^{20} of structure (II), in other words preferably R^{17}, R^{27}, R^{37}, R^{45}, R^{44}, R^{13}, or R^{20} is -QP.

A further embodiment of the present invention provides a method of synthesis of a copolymer conjugate (I) by reacting a reactive polymer (VI)



(VI)

wherein P is a polymeric backbone as defined above, L is a pendent group m is an integer of between 1 and 10,000, U is a leaving group; with a heterocylic compound (VII)



(VII)

in which one and only one of R^{61} to R^{69} is R^{70}, a group reactive with -LU, to form a covalent bond with L by displacement of U, whereby the group -Q- is formed, and the remaining groups R^{61} to R^{69} are identical to groups R^1 to R^9 respectively or are protected precursors thereof.

Preferably R^{70} comprises an amino group, an amino C_{1-18}-alkyl group, a carboxylic group or a hydroxyl group or a mixture thereof. U preferably comprises an activated leaving group such as p-nitrophenol, tosyl, I, Br and is most preferably joined to a terminal carbonyl group of L. Most preferably R^{70} and LU react to form a peptide bond and displace U. R^{70} preferably is a primary or secondary amino group (where it is joined to a ring carbon) or an aminoalkyl group and LU is preferably a group $-R^{71}$-COU. Preferred leaving groups U are p-nitrophenyl or R^{71} is a divalent group, preferably having at least 6 up to about 50 atoms in the chain between the bonds joining it to P and the carbonyl group. Most preferably R^{71} is an oligopeptide group.

Suitable water-soluble reactive polymers (VI) having functional pendent moieties -LU of a suitable molar content are readily available and, for instance, are of the type used to form polymer therapeutics as described above. One particularly convenient class of reactive polymers are the HPMA copolymers with methacrylic acid with pendent oligopeptide groups joined via peptide bonds to the methacrylic acid with activated carboxylic terminal groups such as paranitrophenyl derivatives.

In the process, the heterocyclic compound of the formula (VII) is used in a suitable amount to give the desired degree of derivatisation of the polymer. Where 100% derivatisation is desired the heterocyclic compound is used in stoichiometric amounts or higher for complete reaction of all -LU groups. Where less than complete derivatisation of those groups takes place, residual reactive groups -LU are generally blocked, after the reaction with heterocyclic compound, to deactivate the groups. Blocking reagents suitability comprises the same group as the functional group of R^{70}, ie., a primary or secondary amino group.

Generally the extent of derivatization of groups LU by the compound (VII) is in the range of 1 to 100%, preferably 10 to 99%, for instance at least 50%.

Heterocyclic compounds of the general formula (VII) have been described in the prior art and may be synthesised using techniques analogous to those of the prior art. For instance compounds in which R^{70} is an amino group have been described in, *inter alia*, WO-A-9200315, whilst compounds in which R^{70} is an aminoalkyl group have been desribed in EP-A-0209511, FR-A-2527209 and US-A-4310667, the substituent being on a ring nitrogen of the pyridinium ring, whilst amino alkylaminocarbonyl substituents at the 1-carbon atom are described in EP-A-0591058. Similarly, in EP-A-0608876, derivatives with primary amine groups available for reaction with activated carboxylic compounds $P(R^{71}COU)m$, are amino acid esters of 9-hydroxylated ellipticine. Other useful amino-group containing starting compounds may be made using the synthetic processes of the type described in EP-A-0402232, using ring formation with starting reagents having protected alkyl amino substituents at an appropriate position.

Cleavage of the linker of the conjugate preferably results in release of bioactive heterocycle. The linker preferably comprises at least one cleavable peptide bond. Preferably the linker is an enzyme cleavable oligopeptide group preferably comprising sufficient amino acid units to allow specific binding and cleavage by a selected cellular enzyme. Preferably the linker is at least two amino acids long, more preferably at least three amino acids long.

A further embodiment of the invention is the provision of a method of selectively degrading a polymer-bioactive heterocycle conjugate as defined herein before, comprising the steps of:

a) introducing the polymer-bioactive heterocycle conjugate to a lysosomal environment,

b) cleaving said polymer.

Yet a further embodiment of the invention is the provision of method for releasing a bioactive heterocycle comprising the steps of

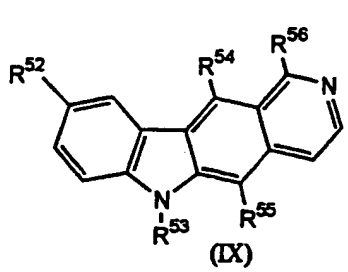
a) introducing the polymer-bioactive heterocycle conjugate to a lysosomal environment,

b) cleaving the bioactive agent from the polymer by acid or enzymic hydrolysis,

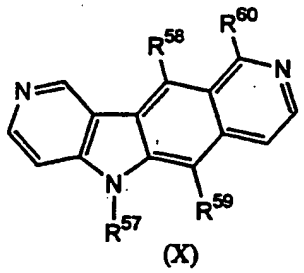
The present invention also envisages the production of compositions and pharmaceutical compositions comprising the polymer-heterocyclic bioactive agent conjugate of the present invention.

Detailed Description of the Invention

Particularly preferred bioactive heterocyclic agents utilised in the present invention are (IX) and (X), shown below



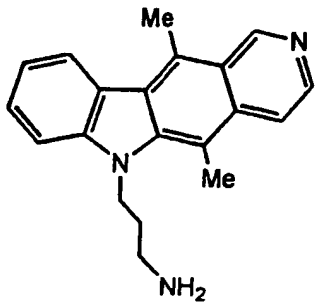
(IX)



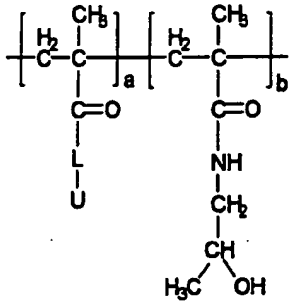
(X)

wherein R^{52} is selected from the group consisting of hydrogen, hydroxy, C_{1-4} alkoxy or C_{2-12} acyloxy, most preferably hydrogen or hydroxy. R^{53}, R^{56}, R^{60} and R^{57} are selected from the group consisting of hydrogen, alkylaminoalkane-1, 2-diyl, dialkylaminoalkane-1, 2-diyl, aminoalkane-1, 2-diyl, alkoxy and hydroxy, preferably aminoalkane-1, 2-diyl, most preferably aminoethyl, aminopropyl, aminobutyl, aminopentyl and aminohexyl. R^{54}, R^{55}, R^{58} and R^{59} are selected from the group consisting of H, C_1 - C_{12} alkyl, C_6 - C_{12} aryl, C_1 - C_{12} aralkyl, C_6 - C_{12} cycloalkylene, preferably C_1 - C_8 alkyl, most preferably methyl.

In a particularly preferred embodiment of the present invention the heterocyclic group is 6-(aminoalkyl)-ellipticine, most preferably 6-(3-aminopropyl)-ellipticine (APE) shown below



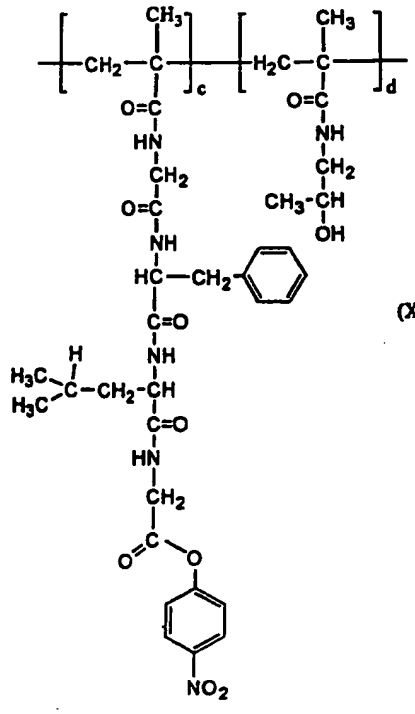
In a preferred embodiment the polymeric backbone comprises a hydroxyalkyl(alk)acrylamide methacrylamide copolymer, most preferably a copolymer of hydroxypropyl(meth)acrylamide copolymer (HPMA). The HPMA prior to attachment of the ellipticine group has the structure (XI)



(XI)

wherein L and U are as defined above. a can be in the range of 0.01-100 and b can be in the range 0-99.99. a is preferably in the range of 0.04-20 and b is preferably in the range 80-99.96. Preferably L is an oligopeptide group containing between 2 and 10 peptide moieties, most preferably 3 or 4.

In a most preferred embodiment, the preferred embodiment L is a Gly-Phe-Leu-Gly- linkage. Preferably U is an ONp group, wherein Np is a p-nitrophenyl group. The HPMA copolymer GFLG-ONp polymer preferably has the structure (XII)



(XII)

wherein c and d are as defined for a and b above. Preferably c is in the range 0.3 to 15 and d is in the range of 99.7 to 85.

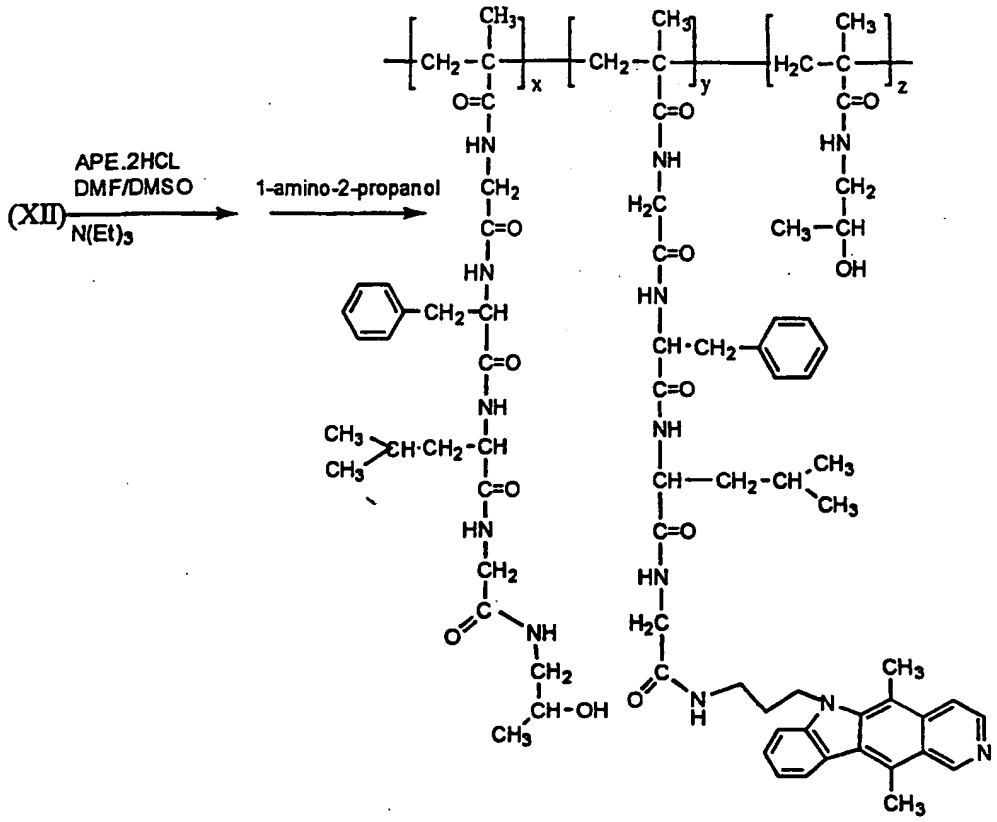
Whilst the heterocyclic bioactive agent conjugate may rely for its localisation at a solid tumour primarily upon EPR, it may be desirable to attach ligands allowing active targeting.

The conjugate of polymer and bioactive agent moiety preferably has a molecular weight in the range 100 D to 800 kD, more preferably in the range 15 kD to 40 kD.

In a preferred embodiment 6-(3-aminopropyl)-ellipticine (APE) is linked to a copolymer via pendent, lysosomally labile peptide side-chains. The bioactive agent is linked to the polymer using aminolysis of carboxyterminal p-nitrophenyl ester groups (Ulbrich et al., 1996[11]).

As the % loading of APE per equivalent of activated ester group present on HPMA can be tailored depending on the utility, the APE can be loaded in the required quantity, preferably in the range 10-100% of available peptide linker. The unloaded linkers can be blocked using an agent such as 1-amino-2-propanol or further derivatized.

In the particularly preferred embodiment of the invention, the preparation of the polymer-bioactive agent conjugate takes place via a 2 step process as shown in Scheme 1 below. The initial step is the addition of APE.2HCl, in a specifically selected equivalence ratio, to a polymer having the structure (XII) as defined above. This results in a stricly defined proportion of the total available peptide linkers being loaded with APE. The second step involves blocking or further derivatization of the remaining unloaded reactive pendent oligo peptide groups with 1-amino-2-propanol.



Scheme 1

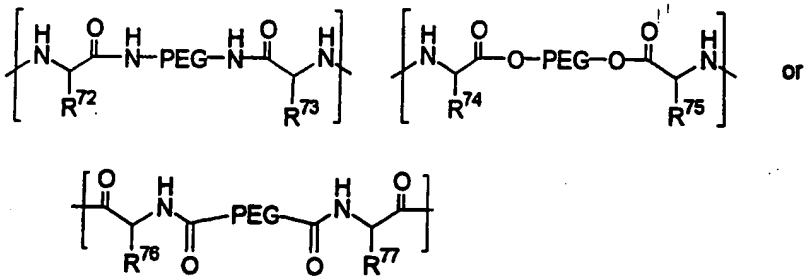
In the above scheme, it can be seen that x+y=c and z=d.

In the preferred embodiment, the N-C bond that is created when ONp is displaced is the bond that is cleaved by the cellular proteinases.

HPMA copolymer-aminopropylellipticine (APE) displays considerably increased solubility in aqueous solution compared to free drug, and obviates the hemolytic effects of low molecular weight ellipticine, believed to be due to charge-dependent association of the ellipticine with phospholipids in cell membranes, as determined for red cell ghosts (Lee, 1976[12]).

In another preferred embodiment the polymeric backbone comprises derivatised polyethyleneglycol, most formed preferably from amine derivatised polyethyleneglycol by reaction with amino acids or oligopeptide with reactive side chains.

Most preferably the derivatised polyethyleneglycol comprises a structure comprising a repeating unit selected from the group consisting of



or

wherein PEG is a polyethyleneglycol, and wherein one of R^{72} and R^{73}, R^{74} and R^{75}, and R^{76} and R^{77} is a group LU as defined above and the remaining groups R^{72} to R^{77} are selected from, hydrogen, C_{1-6} alkyl, C_{6-12} aryl, C_{7-13} alkaryl and C_{7-13} aralkyl groups, any of which may be unsubstituted or substituted by amine, hydroxyl, alkoxy, acyl, acyloxy, acylamino, aminocarbonyl, carboxylic, or oligopeptide groups.

Brief description of the drawings

Figure 1 is the HPLC profile of an HPMA-GFLG-APE conjugate after hydrolysis by HCl, (described in Example 5).

Figure 2 shows size exclusion chromatograms of HPMA-GFLG (A), HPMA-GFLG-APE (B) and methanol (C), as described in Example 6;

Figure 3 shows the results of Example 8, the key for which is

Example 1.1.1 WT	Example 2 WT
Example 1.2.1 WT	Example 2 WOT
Example 3 WT	Example 3 WOT
Example 1.1.1 WOT	Example 1.2.1 WOT

wherein WT is with tritosomes and WOT is without tritosomes.

Figures 4 and 5 show the result of Example 9. The key to Figure 4 is as follows:

- APE % lysis 1 hour
- Ellipticine % lysis 1 hour

The key to Figure 5 is as follows:

—▲— Example 1.2.1 24 hr incubation
—▫— Example 2 1 hr incubation
—■— APE at 1 hr incubation
—▲— Example 1.2.1 1 hr incubation
—●— Example 2 24 hr incubation
—◇— Example 1.1.1 1 hr incubation
—◆— Example 1.1.1 24 hr incubation

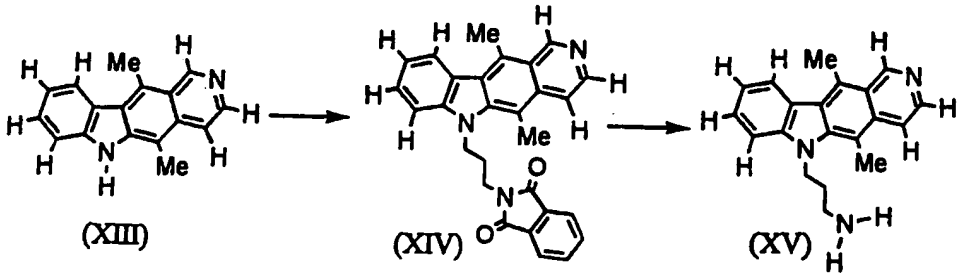
The following examples illustrate preferred embodiments of the invention.

Examples

General

6-(3-aminopropyl)-ellipticine, APE.2HCl was obtained from the National Cancer Institute, Washington, U.S.A and prepared by the following method: Ellipticine (XIII) was treated with sodium hydride (NaH) (20% excess) and bromopropylphthalimide (20% excess) in dimethyl formamide containing 1 equivalent of hexamethylphosphoric triamide (HMPT) at room temperature for 6.5 hours. This afforded the phthalimidopropylellipticine derivative (XIV), m.p. 225-230° (yield 64%). To remove the phthaloyl group, (XIV) was treated with sodium hydroxide in aqueous methanol at 50° for 2 hours, followed by treatment with hydrochloric acid on a steam bath for 2 hours. This afforded the 6-(aminopropyl)-derivative (XV) in 54% yield. Analysis defined the compound as a dihydrochloride. The reaction scheme is shown below in scheme 2.

An analogous method can be used to produce the 9-methoxy-6-(3-aminopropyl)-ellipticine derivative.



Scheme 2.

Dry solvents were supplied by Aldrich and dispensed under argon. Chemicals were obtained from Aldrich, Fischer Chemicals or BDH Ltd., unless otherwise stated.

Example 1. Preparation of HPMA copolymer-GFLG(5mol%)-APE

HPMA copolymer-GFLG-ONp (either 1, or 5 mol% peptidyl side chains; Mw~30,000Da and Mw/Mn = 1.2-1.4) was prepared using methods previously described (Rejmanova et al. 1977[13]), the content of displaceable activated ester being calculated from the extinction coefficient of bound 4-nitrophenol at 274nm in DMSO.

Small scale pilot preparations (1-5 mg of polymer) of conjugates using aminolysis, were followed by 4-nitrophenol release over time on dropwise addition of triethylamine to a mixture of HPMA copolymer and APE.2HCl in dimethyl formamide/dimethyl sulphoxide until optimal conditions were established for a medium scale preparation.

HPMA-Gly-Phe-Leu-Gly-p-nitrophenol ester (5mol%) (1g) was dissolved with stirring in dry dimethyl formamide (25ml). 6-(3-aminopropyl)-ellipticine dichloride, APE.2HCl (84mg, 0.75 equivalent) was dissolved in dry dimethyl sulphoxide (17ml). For the 1% pHPMA-GFLG-ONp the proportions of the starting polymer and APE were adapted appropriately. The solutions were mixed, a small aliquot of each (5-10µl) being retained for thin-layer chromatography. A dilute solution of dry triethylamine in dry dimethyl formamide (1:100 v:v, 9.3ml, 3:1 equivalents to APE.2HCl) was prepared for addition in aliquots (500µl at 5min. intervals) to the reaction mixture. After each addition, with swirling, samples (10µl) were taken into a 1ml cuvette containing Dulbecco's phosphate buffered saline (pH 7.0) and the initial absorbance at 400nm noted to follow the release of 4-nitrophenol (UV-Visible Spectrophotometer Shimadzu UV-1601). The reaction mixture, initially pale yellow, decolourises after the first six aliquots of triethylamine then progressively deepens to yellow colour. Measurements of absorbance at 400nm reach a plateau as triethylamine addition is completed, consistent with the displacement of the calculated amount of 4-nitrophenol (0.75 equivalents). The disappearance of unbound APE.2HCl was followed by thin-layer chromatography (on silica gel plates Kieselgel ALU 60 F_{254} from

Merck, using chloroform/methanol/triethylamine 8/1/1 (V/V) as mobile phase). RF APE.2HCL: 0.4, purple fluorescence, 354 nm. Bound APE remains at the origin. RF 4-nitrophenol 0.9 yellow spot visible, dull purple spot, 354nm. The reaction mixture was left overnight in the dark, then quenched with dilute 1-amino-2-propanol (1:00 v:v in dry dimethyl formamide, 2.2 ml) for 1hr. The solvents were evaporated (High vacuum, Javac oil pump, liquid nitrogen trap) at 30°C, and the resulting gum was dissolved in distilled water (60ml) and dialysed against three changes of distilled water over 48 hrs (Spectropor 2000 MWCO, solvent resistant). The contents of the dialysis tubing were freeze–dried to constant weight (750mg). Characteristics of typical products of this synthesis determined by the techniques described in Examples 4 and 5 below are shown in table 1.

Conjugates	Example	%Total APE (w/w conj.)	% impurity (w/w of conj.) APE	Ellipticine	% free APE (w/w total APE)	%Theor. yield
HPMA-GFLG (5mol%)-APE	1.1.1*	2.3	<0.06	<0.10	2.60	25.7
HPMA-GFLG (5mol%)-APE	1.2.1	7.2	<0.02	<0.10	0.28	83.5
HPMA-GFLG (5mol%)-APE	1.2.2	6.0	<0.03	<0.05	0.36	68.9
HPMA-GFLG (5mol%)-APE	1.2.3	5.5	<0.02	<0.05	0.25	62.9
HPMA-GFLG (1mol%)-APE	1.3.1	1.07	<0.08	<0.05	7.74	56.0
HPMA-GFLG (1mol%)-APE	1.3.2	1.20	<0.09	<0.05	7.65	62.9
HPMA-GFLG (1mol%)-APE	1.3.3	1.21	<0.05	<0.05	4.32	63.5

Table 1: Amount of total APE (determined by UV at 295nm), amounts of free APE and ellipticine (determined by HPLC after extraction) present in the different conjugates. * prepared using a method prior to the optimisation discussed before.

Example 2. Preparation of HPMA copolymer-GG(5mol%)-APE

The preparation of the HPMA copolymer with a glycine-glycine peptide linker rather than a GFLG linker is now described. HPMA copolymer-GG-ONp prepared (5 mol% peptidyl side chains; Mw~30,000Da and Mw/Mn = 1.2-1.4) (Rejmanova et al. 1977[13]) (1 equivalent, calculated as 4-nitrophenyl ester) and APE.2HCl (2 equivalents) were dissolved in minimal volumes of dry dimethyl sulphoxide. Triethylamine (2 equivalents) was added to the copolymer solution to neutralise the hydrochloride protons of the APE.2HCl solution which was added dropwise to the reaction mixture. Aminolysis was allowed to proceed for 5 hours. To complete the aminolysis of any unreacted esters, 1-amino-2-propanol (2 equivalents) were added to the reaction mixture (1 hour). The dimethyl sulphoxide was removed at 30°C under high vacuum and the residue redissolved in distilled water. The clear solution, comprising conjugate 4-nitrophenol and unreacted 1-amino-2-propanol and/or unreacted APE.2HCL was dialysed against distilled water (MWCO 5,000, solvent resistant grade, cellulose ester, Spectrapor, USA, Pierce and Warriner, U.K.). For small scale preparations the void volume molecular weight fraction from PD10 (Sephadex G25) column gel chromatography in water was freeze-dried. For larger scale preparations the column step was omitted.

Conjugate	Example	%Total APE (w/w conj.)	% impurity (w/w of conj.) APE	Ellipticine	% free APE (w/w total APE)	%Theor. yield
HPMA-GG (5mol%)-APE	2.	3.8	<0.01	<0.05	0.25	45.7

Table 2: Amount of total APE (determined by UV at 295nm), amounts of free APE and ellipticine (determined by HPLC after extraction) present in HPMA copolymer-GG-APE

Example 3. Preparation of HPMA copolymer-GFLG(10mol%)-APE

HPMA copolymer-GFLG-ONp was prepared (10 mol% peptidyl side chains; Mw~30,000Da and Mw/Mn = 1.2-1.4) (Rejmanova et al. 1977[13]), the content of displaceable activated ester being calculated from the extinction coefficient of bound 4-nitrophenol at 274nm in DMSO. HPMA copolymer-

GFLG-ONp (1 equivalent, calculated as 4-nitrophenyl ester) and APE.2HCl (2 equivalents) were dissolved in minimal volumes of dry dimethyl sulphoxide. Triethylamine (2 equivalents) was added to the copolymer solution to neutralise the hydrochloride protons of the APE.2HCl solution which was added dropwise to the reaction mixture. Aminolysis was allowed to proceed for 5 hours.

To complete the aminolysis of any unreacted esters, 1-amino-2-propanol (2 equivalents) were added to the reaction mixture (1 hour). The dimethyl sulphoxide was removed at 30°C under high vacuum and the residue redissolved in distilled water. The clear solution, comprising conjugate 4-nitrophenol and unreacted 1-amino-2-propanol and/or unreacted APE.2HCL was dialysed against distilled water (MWCO 5,000, solvent resistant grade, cellulose ester, Spectrapor, USA, Pierce and Warriner, U.K.). Overall yields based on polymer weight were as follows:

Conjugate	Example	%Total APE (w/w conj)	% impurity (w/w of conj.) APE	Ellipticine	% free APE (w/w total APE)	%Theor. yield
HPMA-GFLG (10mol%)-APE	3.	7.0	<0.01	<0.05	0.11	47.5

Table 3: Amount of total APE (determined by UV at 295nm), amounts of free APE and ellipticine (determined by HPLC after extraction) present in HPMA copolymer-GFLG-APE (10 mol%)

Example 4. HPLC method for analysis of free APE in HPMA copolymer APE

Low molecular weight impurities and free APE in conjugates HPMA-APE were determined by extraction with chloroform/isopropanol and HPLC assay (see tables 1, 2 and 3). Samples of HPMA-APE conjugates (1 to 5mg) or APE (1-10mg) for calibration were dissolved in 900µl of water. 100µl of Doxorubicin (0.3g/l) were added (internal standard). pH of samples was adjusted to 8.5 with 200µl of ammonium formate buffer (1M) and samples were extracted with 8 ml of a mixture chloroform/2-propanol (80/20). After evaporation of the solvents under nitrogen, the dry residue was dissolved in 200 µl of methanol/water 60/40 (V/V) and amount of APE determined by

HPLC using a Column Bondapak C18 (150mm x 3.9 mm), a pump (LKB Bromma 2150 HPLC pump) which delivers the mobile phase (methanol/water 60/40 (V/V) pH 2.2 adjusted with o-phosphoric acid) at 1 ml/min. The UV detection of APE, ellipticine and doxorubicin was achieved by a fixed wavelength filter at 307 nm (Spectroflow 783 Kratos analytical).

The results are shown in the tables 1-3 above. The area under the curve is used to convert the results to concentrations using the calibration curve.

Example 5. HPLC method for analysis of total APE in HPMA copolymer APE

1ml of HCl 6N was added to 900µl of a solution of HPMA copolymer-APE. The mixture was then incubated at 100°C during 3 hours, the HCl was then neutralised by adding 1ml of NaOH solution (6N) and 1.5ml of ammonium formate buffer (1M), and 100µl of doxorubicin solution (0.3g/l, internal standard) were added to this mixture. The free drug (after hydrolytic cleavage of the covalent bond between the copolymer and APE) was extracted by a mixture chloroform/2-propanol 80/20 (V/V); after evaporation of the solvents under nitrogen, the dry residue was dissolved in 200µl of methanol/water 60/40 (V/V) and the amount of APE was determined by HPLC using the same system as described in example 4 previously, including the calibration. This method determines the total quantity of APE in the initial polymer-bioactive agent conjugate. The results for the products of the Examples 1-3 are shown in tables 1-3 above. A typical HPLC profile for one of the batches made in examples 1-3 above is shown in Figure 1.

Example 6. Analyses of HPMA copolymer-APE conjugates by Size Exclusion Chromatography

HPMA copolymers-APE conjugates, and for comparison the HPMA-GFLG precursor after hydrolysis of the ONp ester linkage, were analysed by size exclusion chromatography (SEC) performed using a system composed of two TSK-gel column in series (G3000 PW followed by G2000 PW) with a guard column (ProgelTM PWXL). The detection was achieved using a Refractometer (153 Refractive index detector Gilson) and a UV-visible detector (UV Savern Analytical SA6504) in series. The mobile phase used was a Tris buffer (Tris 0.05M, NaCl 0.5M) delivered at 1ml/min by a pump

(Jasco PU-980). Figure 2 shows size exclusion chromatograms for the conjugates and polymer comparison, showing refractive index (RI) (y axis) against time (x axis).

Example 7. Comparative solubilities of APE.2HCl and HPMA-Gly-Phe-Leu-Gly-APE (5mol%) in phosphate buffered saline.

From a stock solution of APE.2HCl in dimethyl sulphoxide (DMSO), triplicate volumes were pipetted (Gilson-range 0-50µl) into 0.1 M phosphate buffered saline (PBS) to determine the absorbance at 296 nm for a concentration range of APE.2HCl of 0-5µg/ml. A saturated solution of APE.2HCl in PBS (5ml) was obtained at 37°C, the pH being adjusted to 7.4 by addition of small aliquots of 0.1M sodium hydroxide. After equilibration at 37°C for 10 min. and rechecking the pH, the warm solution was filtered through a 0.45 micron filter attached to a syringe. Volumes (10µl) were diluted in triplicate in 1ml PBS maintained at 37°C and the absorbance at 296nm was measured. The original saturated solution was allowed to cool to room temperature (19°C) and determinations repeated as before at room temperature. In a similar experiment, in which HPMA-Gly-Phe-Leu-Gly-APE (5mol%) product of example 1.3.3 (50mg) was dissolved in PBS (2ml) at 37°C with pH adjustment and equilibration for 10 min., a clear solution was obtained which was nevertheless filtered (to allow for equivalent losses) and treated as before to determine the concentration of dissolved APE bound to the polymer.

At room temperature, a saturation was reached for 44.5µg of APE in 1ml of PBS at pH 7.42 at 37°C, the saturated solution contain 62.4µg/ml of APE in PBS at pH 7.42. At a concentration of 625.2µg/ml in APE equivalent, the conjugate HPMA-GFLG(5mol%)-APE (5.5% w/w) is still soluble at 37°C. So the solubility of APE is at least ten times greater when it is coupled on the HPMA copolymer.

Example 8. Degradation of HPMA copolymers-APE by tritosomes (Rat liver lysosomes)

Preparation of tritosomes and determination of the activity of rat lysosomal enzymes: Rat lysosomal enzymes, tritosomes, were prepared according to the method described by Trouet (1974[14]) and the protein

content was determined using bicinchonninic acid assay, which consists of comparing the UV absorbance at 550nm of solutions of tritosomes at different concentrations containing bicinchonninic acid and copper sulfate with the absorbance of solutions of bovine serum albumin at different concentrations containing also bicinchonninic acid and copper sulfate. The protein content of the tritosomes was found to be 1.697mg/ml. The proteolytic activity was determined as the release of p-nitroaniline from N-benzoyl-Phe-Val-Arg-p-Nitroanilide by the tritosomes (Trouet, 1974[14]). Activity of the proteases was found to be 25 nM/min/mg protein. This test is carried out to validate that an individual preparation contains appropriate enzymatic activity against a standard substrate.

Incubation of HPMA copolymer-APE conjugates with tritosomes A mixture of 100µl of sample (from solutions of HPMA copolymer-APE conjugates at 5mg/ml or from APE solutions between 0.01 to 0.1 g/l), 100µl EDTA (10mM), 100µl GSH (50mM), 400µl of citrate-phosphate buffer (0.1M citric acid, 0.25M Na_2HPO_4, 0.2% Triton X-100, pH 5.5), 100µl of doxorubicin (0.3g/l, internal standard) and 200µl of tritosomes was incubated at 37°C. Tritosomes were added last and the tubes (3 replicates) were thoroughly mixed. 100µl aliquots of reaction mixture were taken at time 0, 1, 2, 4, 6, and 10 hours, immediately frozen in liquid nitrogen and stored frozen in the dark until processed by HPLC

HPLC evaluation of degradation The 100µl samples were mixed with 800µl of water and 100µl of an ammonium formate buffer, and then APE was extracted by 5ml of a mixture chloroform/2-propanol 80/20 (V/V). The organic fraction was evaporated to dryness under nitrogen and the dry residue was dissolved in the mobile phase used for HPLC, which is methanol/water 60/40 (V/V), pH 2.2 adjusted with o-phosphoric acid.

During all this procedure, samples have to be kept on ice to minimise APE degradation within the organic phase, which otherwise takes place at room temperature to give ellipticine.

Figure 3, shows the total amount of APE released against time from the conjugates HPMA-GG-APE (product of example 2) and HPMA-GFLG-APE (Examples 1.1.1, 1.2.1 and 3). The product of Example 1.1.1 contains

2.3 weight% of APE, and 2.6% of the total APE is free. The results for APE release are based on the total APE detected, ie., including the inherent free APE. All these conjugates were incubated at 37°C either in the presence of tritosomes (WT) or without tritosomes (WOT) at a concentration of 46µg/ml in APE.

The conjugate HPMA-GG-APE did not release APE, neither did the other conjugates in the absence of enzyme.

The two HPMA copolymer-GFLG-APE conjugates which have different amount of spacer arms batch, Example 1.2.1 (5mol%) and Example 3 (10mol%) but which have the same total amount of APE bound, released APE at the same rate. The conjugate HPMA-GFLG(5mol%)-APE (Example 1.1.1) released 80% of the bound APE. In summary, the conjugates release drug in relation to the the peptide spacer used and the amount of APE bound and the content of peptidyl side chains.

Example 9. Evaluation of the hemolytic properties of HPMA copolymer-APE, ellipticine and free APE

HPMA-GFLG-APE (Product of Examples 1.1.1 and 1.2.1), HPMA-GG-APE (Example 2), APE and ellipticine were incubated at 37°C with rat erythrocytes (final drug concentration between 5mg/ml to 1µg/ml) for 1 and 24 hours. The cell debris was then removed by centrifugation and the hemoglobin released through the red blood cell (RBC) lysis assessed by use of a micro-titre plate reader at 550nm. The readings are calculated as RBC lysis percent compared with total hemolysis by Triton X-100. The results for ellipticine and APE are shown in Figure 4, whilst the results for the conjugates (and APE for comparison) are shown in Figure 5.

It can be seen (Figure 4) that ellipticine and APE are very hemolytic. Indeed APE is significantly more lytic than ellipticine (concentration of 50% of red blood cell lysis is of the order of 35 µg/ml for APE and 600 µg/ml for Ellipticine).

When HPMA copolymer-APE conjugates were incubated with RBC no hemolysis was observed over the concentration range (below 14 mg/ml in conjugates and between 0.001 and 0.4g/l in APE equivalent) studied at 1h and also after the longer incubation time of 24h (Figure 5).

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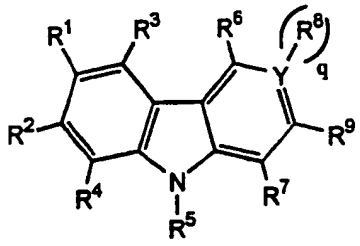
Claims

1. A polymer-bioactive heterocycle conjugate, wherein the bioactive heterocycle conjugate has the structure (I)



(I)

wherein Y is either nitrogen or carbon, each of R^1 to R^9 are selected from the group consisting of QP, hydrogen, hydroxyl, -$CONH_2$, cyano amino, halogen, glycosyl, (di)alkyl amine, C_{1-4} alkoxy, C_{1-12} alkyl, C_1 - C_{12} alkenyl, C_6 - C_{30} aryl, C_7 - C_{30} aralkyl, C_{7-30} alkaryl, C_3 - C_{30} cycloalkyl, C_{2-6} alkanoyloxy and C_{7-10} aralkanoyloxy groups any of which alkyl or aryl groups may be unsubstituted or substituted with a group selected from the the group consisting of carboxy, amine (including (di)alkyl amine) acyl, acyloxy, and acylamino, alkoxy, hydroxy; R^1 and R^2 or R^1 and R^3 may together with the carbon atoms to which they are joined form an aromatic 6-membered substituted or unsubstituted carbocyclic or heterocyclic ring or, if Y is carbon, R^6 and R^8, or R^8 and R^9 may, together with the carbon atoms to which they are joined, form an aromatic carbocyclic or heterocyclic 6-membered ring, provided there is at least one 6 membered nitrogen containing heteroaromatic ring in the molecule, and provided that one and only one of R^1 to R^9 is -QP, Q is a linker group and P is a polymer having a molecular weight in the range 100D to 800KD which is water-soluble; when Y=carbon, q=1 and when Y=nitrogen q=0 or 1.

2. The polymer-bioactive heterocycle conjugate according to claim 1, wherein the polymer is synthetic.

3. The polymer-bioactive heterocycle conjugate according to claim 1 or 2, wherein the polymer is a hydroxypropyl(meth)acrylaminde-methacrylic acid copolymer.

4. The polymer-bioactive heterocycle conjugate according to any preceding claim, wherein R^4, R^6, R^9, and R^7 are selected from the group consisting of hydrogen, hydroxyl, and C_1 - C_{12} alkyl, preferably hydrogen, methyl, ethyl, propyl and butyl, most preferably hydrogen or methyl.

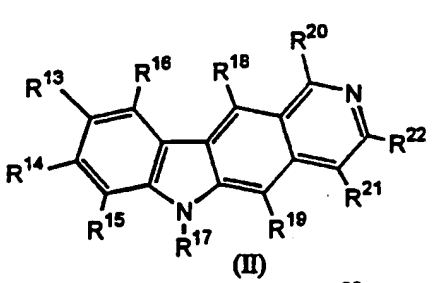
5. The polymer-bioactive heterocycle conjugate according to any preceding claim, wherein Y is carbon, R^6 and R^7 are each methyl, R^1 is hydrogen, hydroxy or acyloxy, R^2 and R^3 are each hydrogen.

6. The polymer-bioactive heterocycle conjugate according to any preceding claim, wheren R^5 is QP.

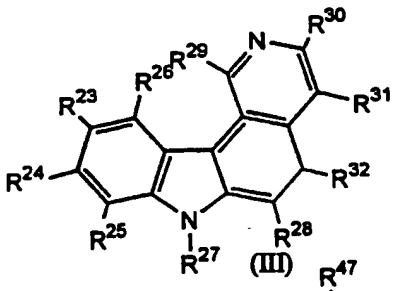
7. The polymer-bioactive heterocycle conjugate according to any preceding claim, wherein QP comprises at least one cleavable peptide bond.

8. The polymer-bioactive heterocycle conjugate according to any preceding claim, wherein QP comprises an acid hydrolysable linker, preferably a cis-aconityl group.

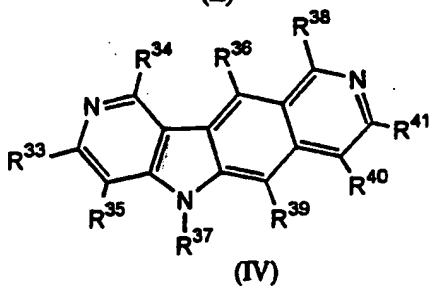
9. The polymer-bioactive heterocycle conjugate according to claim 1, wherein the bioactive heterocycle conjugate has a structure selected from the group consisting of



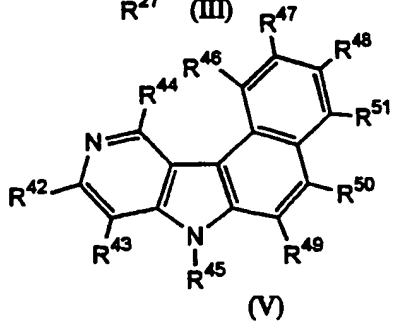
(II)



(III)



(IV)



(V)

wherein R^{13}-R^{51} are selected from the respective groups as defined for R^1-R^9.

10. The polymer-bioactive heterocycle conjugate according to claim 9, wherein the bioactive heterocycle conjugate preferably has the structure (II).

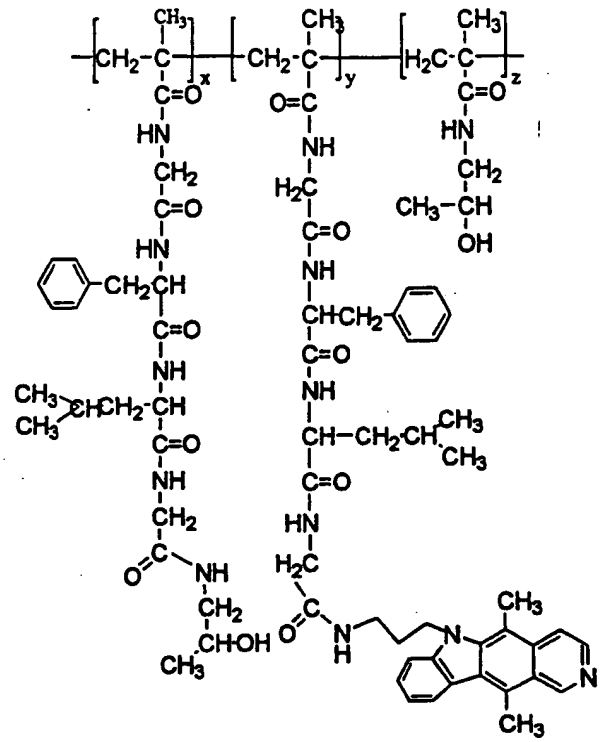
11. The polymer-bioactive heterocycle conjugate acording to claim 10, wherein R^{18} and R^{19} are methyl, R^{13} is selected from the group consisting of hydrogen, hydroxyl, alkoxy and alkanoyloxy, preferably hydrogen, R^{14}, R^{15}, R^{16}, R^{20}, R^{21} and R^{22} are hydrogen; and R^{17} is QP.

12. The polymer bioactive heterocycle conjugate according to claim 9, wherein the bioactive heterocycle conjugate has the structure (III), R^{23} is hydroxy or alkoxy, preferably methoxy. R^{24} to R^{26} and R^{28} to R^{32} are all hydrogen and R^{27} is QP.

13. The polymer-bioactive heterocycle conjugate according to claim 9, wherein the bioactive heterocycle conjugate has the structure (V), R^{48} is hydroxyl or C_{1-4} alkoxy, R^{44} is hydrogen or substituted C_{2-4} alkyl amino, such as (3-N, N-dimethylamino)propyl amino and R^{45} is QP.

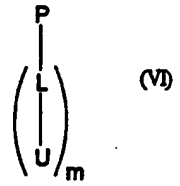
14. The polymer-bioactive heterocycle conjugate according to claim 11, wherein Q comprises an oligopeptide, preferably a glycine-phenylalanine-leucine-glycine linkage.

15. The polymer-bioactive heterocycle conjugate according to claims 11 or 14 wherein the polymer-bioactive heterocycle conjugate comprises the structure



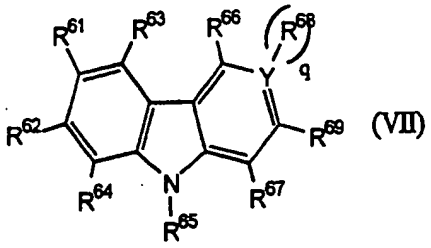
wherein x is in the range 0.01-100, y is in the range 0-99.99 and z is in the range of 0-99.99.

16. A method of synthesis of a copolymer conjugate as defined in any preceding claim, by reacting a reactive polymer (VI)



(VI)

wherein P is a polymeric backbone as defined above, L is a pendent group m is an integer of between 1 and 10,000, U is a leaving group; with a heterocylic compound (VII)



(VII)

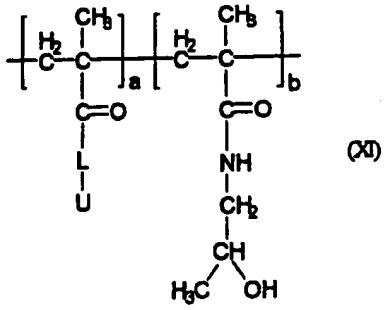
in which one and only one of R^{61} to R^{69} is R^{70}, a group reactive with -LU, to form a covalent bond with L by displacement of U, whereby the group -Q- is formed, and the remaining groups R^{61} to R^{69} are identical to groups R^1 to R^9 respectively or are protected precursors thereof.

17. A method according to claim 16, wherein R^{70} comprises an amino group, an amino C_{1-18}-alkyl group, a carboxylic group or a hydroxyl group or a mixture thereof, U comprises an activated leaving group such as p-nitrophenol, tosyl, I, Br and is preferably joined to a terminal carbonyl group of L.

18. A method according to claim 16 or 17, wherein R^{70} preferably comprises a primary or secondary amino group or an aminoalkyl group and LU is preferably a group $-R^{71}-COUR^{70}$, and reacts with LU to displace U and form a peptide bond.

19. A method according to claim 18, wherein R^{71} is an oligopeptide group.

20. The method according to any of claims 16 to 19, wherein the $P(LU)_m$ has the structure (XI)



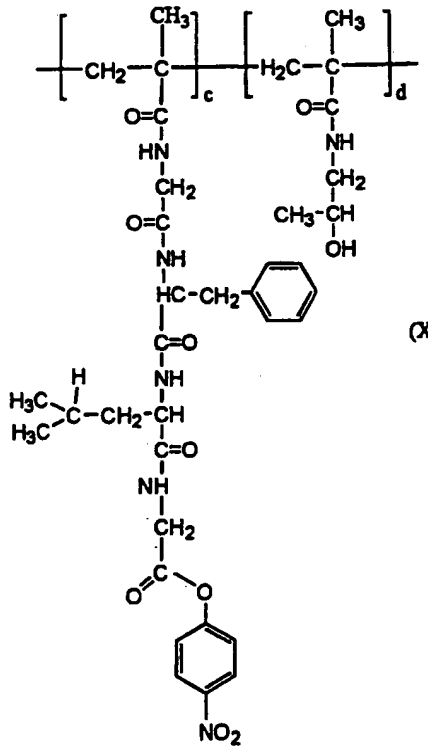
(XI)

wherein a is in the range of 0.01-100 and b is in the range 0-99.99, a is preferably in the range of 0.04-20 and b is preferably in the range 80-99.96.

21. The method according to any of claims 16 to 20, wherein L is an oligopeptide group containing between 2 and 10 aminoacyl moieties, most preferably 3 or 4.

22. The method according to claim 21, wherein L is a Gly-Phe-Leu-Gly group.

23. The method according to claims 16 or 17 wherein the polymer, prior to the attachment of the bioactive heterocycle conjugate comprises the structure (XII)



(XII)

c is in the range of 0.01-100 and d is in the range 0-99.99, c is preferably in the range of 0.04-20 and d is preferably in the range 80-99.96.

24. A method of selectively degrading a polymer-bioactive heterocycle conjugate according to any of claims 1 to 15, comprising the steps of:

a) introducing the polymer-bioactive heterocycle conjugate to a lysosomal environment,

b) cleaving said polymer.

25. A composition comprising at least one polymer-bioactive heterocycle conjugate according to any of claims 1 to 16, and a carrier.

26. A composition comprising at least one polymer-bioactive heterocycle conjugate according to any of claims 1 to 16 and a pharmaceutically acceptable excipient.

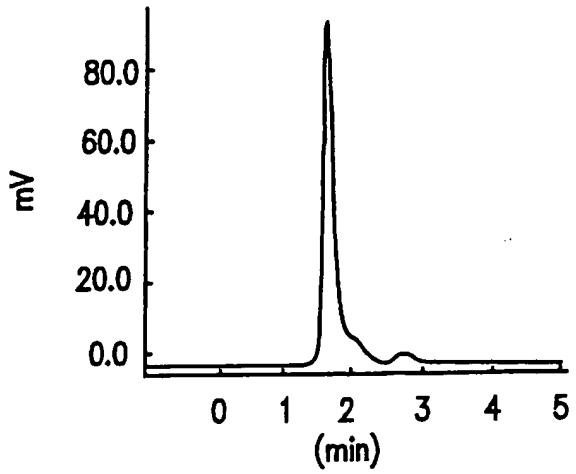


FIG. 1

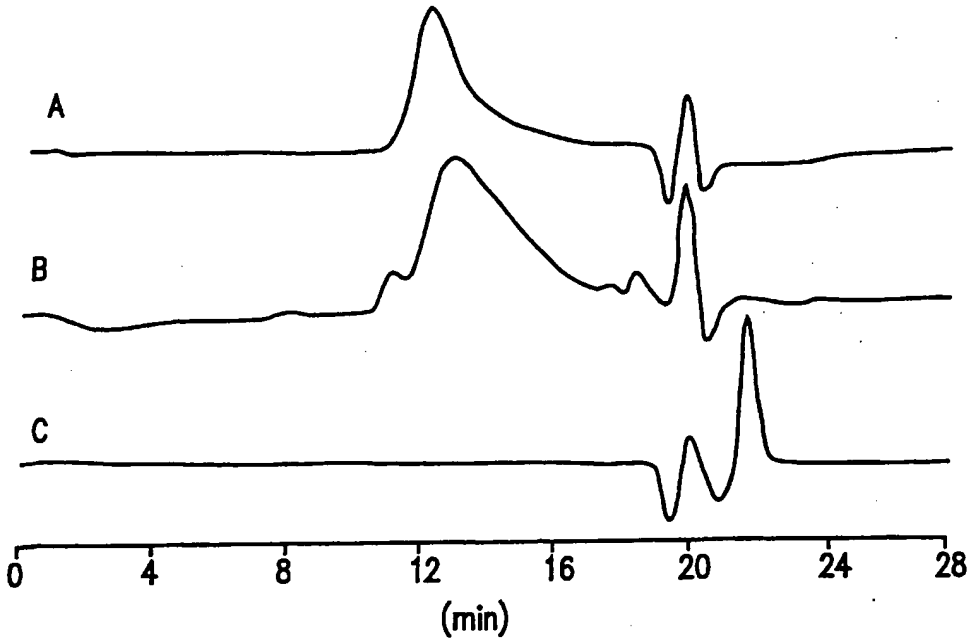


FIG. 2

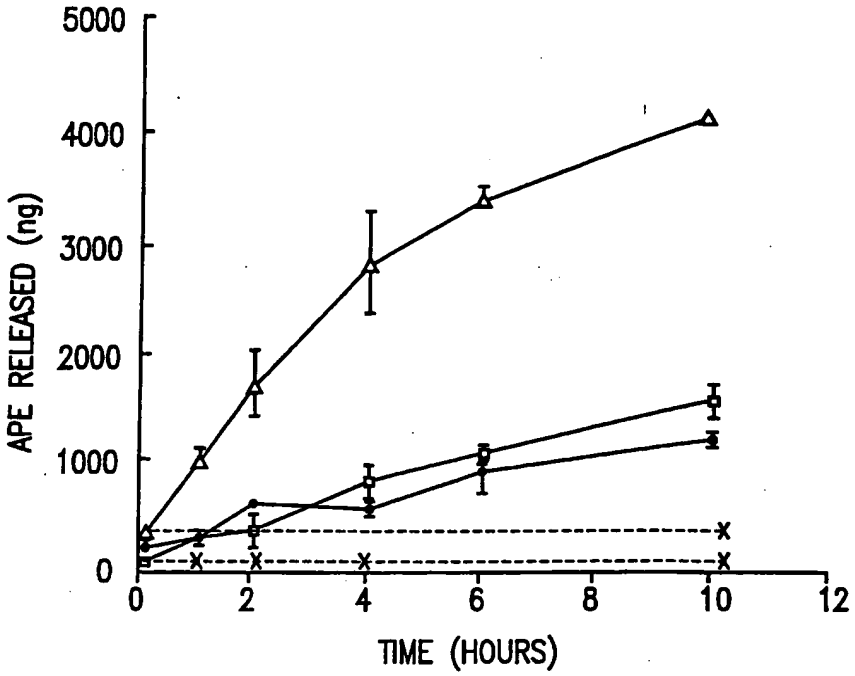

5000
4000
3000
2000
1000
0
APE RELEASED (ng)
0
2
4
6
8
10
12
TIME (HOURS)

FIG. 3

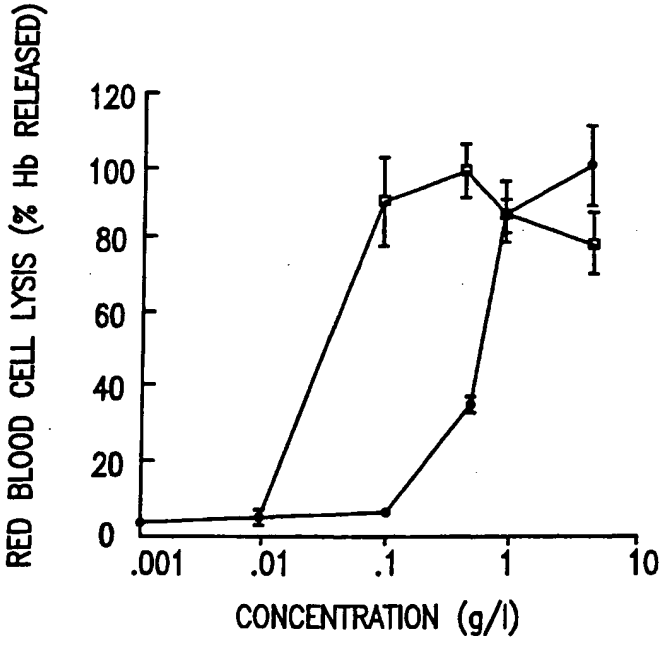

120
100
80
60
40
20
0
RED BLOOD CELL LYSIS (% Hb RELEASED)
.001
.01
.1
1
10
CONCENTRATION (g/l)

FIG. 4

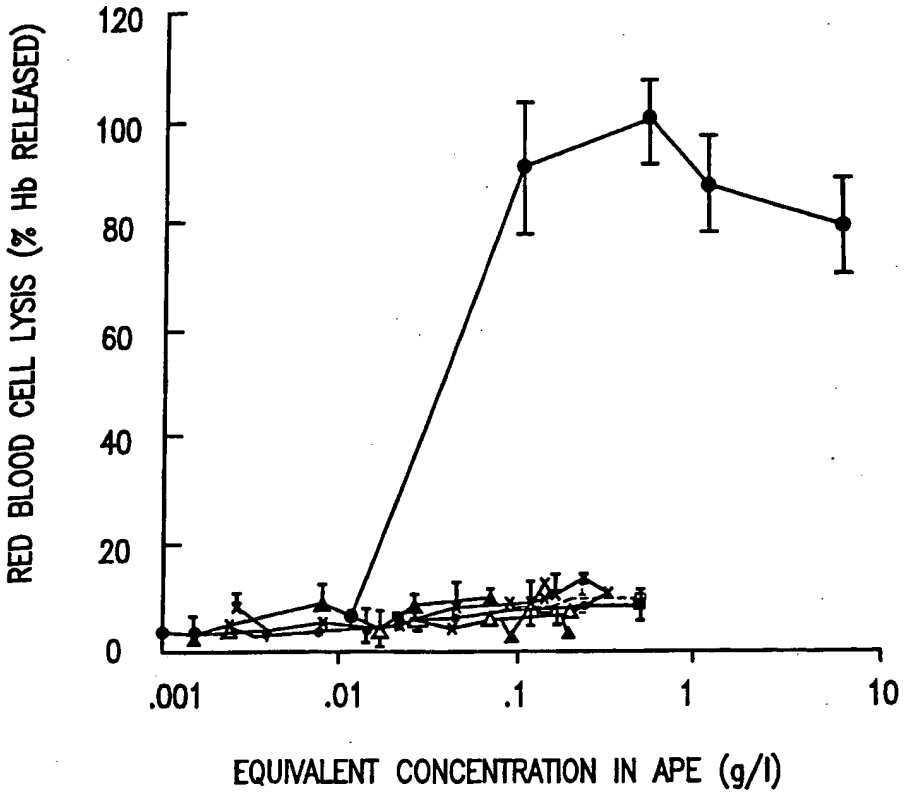

RED BLOOD CELL LYSIS (% Hb RELEASED)
120
100
80
60
40
20
0
.001
.01
.1
1
10
EQUIVALENT CONCENTRATION IN APE (g/l)

FIG. 5

INTERNATIONAL SEARCH REPORT

Inte. .tional Application No
PCT/US 99/27167

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

PAJ, WPI Data, EPO-Internal, CHEM ABS Data, MEDLINE, CANCERLIT, DISSERTATION ABS, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 10304 A (PHARMACIA S.P.A.) 20 April 1995 (1995-04-20)	1-7, 9-14, 16-19, 21-26
Y	examples 6,7	8,15,20
X	US 5 258 453 A (KRINICK NANCY L ET AL) 2 November 1993 (1993-11-02) example 4	1-23
Y	US 5 362 831 A (ANGELUCCI FRANCESCO ET AL) 8 November 1994 (1994-11-08) example 1	1-26
Y	US 5 037 883 A (RIHOVA BLANKA ET AL) 6 August 1991 (1991-08-06) example 1	1-26

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2000	23/08/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Dullaart, A

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INTERNATIONAL SEARCH REPORT

Intl. Jonal Application No
PCT/US 99/27167

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	PAVLA KOPECKOVA ET AL: "CLEAVAGE OF OLIGOPEPTIDE P-NITROANILIDES ATTACHED TO N-(2-HYDROXYPROPYL)METHACRYLAMIDE COPOLYMERS BY GUINEA PIG INTESTINAL ENZYMES" MAKROMOLEKULARE CHEMIE, MACROMOLECULAR CHEMISTRY AND PHYSICS,CH,HUTHIG UND WEPF VERLAG, BASEL, vol. 193, no. 10, 1 October 1992 (1992-10-01), pages 2605-2619, XP000309863 ISSN: 0025-116X page 2616; figure 5	1-26
X	WO 99 17805 A (ANGELUCCI FRANCESCO ;CAIOLFA VALERIA (IT); FACHIN GABRIELE (IT); P) 15 April 1999 (1999-04-15)	1-7, 9-14, 16-19, 21-26
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Y	HASSLER C R ET AL: "Sentinel blood pressure study (dogs) on oral ellipticine NSC-71795" REPORT, NO. BCL-CRH-71795-1;ORDER NO. PB83-194852, 1982, XP000929838 Battelle Columbus Lab.;Columbus; OH; US the whole document	1-26

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-26 in part

Present claims 1-26 relate to an extremely large number of possible compounds, as well as to methods for their preparation, and to compositions containing them. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.
Moreover, the claims contain so many options, variables, possible permutations, that a lack of clarity and conciseness within the meaning of Article 6 PCT arises.
Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the conjugates of ellipticin and HPMA copolymer. Of course, the search has also included methods for their preparation, and compositions containing them.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. .tional Application No
PCT/US 99/27167

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Information on patent family members

Inte. .tional Application No
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